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In-Vitro Studies on Biological Activities of Some *Lichen Extracts*

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ABSTRACT
Lichens are unique symbiotic organisms composed of fungal and photosynthetic partners that produce a wide range of bioactive secondary metabolites with significant pharmacological potential. The present study was undertaken to investigate the phytochemical composition and evaluate the biological activities of selected lichen species collected from Ooty, Tamil Nadu. The lichens were identified using morphological, chemical, and chromatographic techniques, followed by extraction using solvents of increasing polarity such as hexane, ethyl acetate, methanol, and ethanol. Qualitative and quantitative phytochemical analyses revealed the presence of various bioactive constituents including alkaloids, flavonoids, phenols, tannins, terpenoids, steroids, carbohydrates, and anthraquinones. Among these, phenolic and flavonoid compounds were found to be predominant, particularly in methanolic extracts. The biological evaluation demonstrated significant antimicrobial, antioxidant, antidiabetic, and anticancer activities. Methanolic extracts exhibited strong antimicrobial activity with low MIC values and effectively altered microbial growth kinetics. The extracts also showed dose-dependent inhibition of α-amylase and β-glucosidase enzymes, indicating antidiabetic potential. Furthermore, cytotoxic studies revealed selective anticancer activity through induction of apoptosis and cell cycle arrest. Isolation and characterization of bioactive compounds confirmed the presence of pharmacologically important metabolites such as stictic acid, constictic acid, and anthraquinone derivatives. Overall, the findings suggest that lichens are a rich and underexplored source of natural bioactive compounds with promising therapeutic applications in modern medicine.

Keywords
Lichens,
Secondary metabolites,
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Antioxidant,
Antidiabetic,
Anticancer

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Introduction

Lichens are remarkable composite organisms formed by a symbiotic association between a fungal partner (mycobiont) and a photosynthetic partner (phycobiont), typically algae or cyanobacteria [1]. In this association, both partners lose their independent identity and function as a single biological entity. The fungal component constitutes the major portion of the lichen thallus and provides structural support, while the photosynthetic partner synthesizes carbohydrates essential for growth and metabolism. Lichens are widely distributed across diverse ecological habitats, ranging from tropical forests to polar regions, and are capable of surviving under extreme environmental conditions such as desiccation, intense radiation, and temperature fluctuations [9,10]. Their ability to adapt to harsh environments is attributed to the production of unique secondary metabolites, which play a crucial role in protection against environmental stress and microbial attack. Approximately 20,000 lichen species have been reported worldwide, covering nearly 10% of the Earth's terrestrial surface [2,3].

Lichen metabolites are broadly classified into primary and secondary metabolites. Primary metabolites such as proteins, lipids, and polysaccharides are essential for growth and metabolism, whereas secondary metabolites such as depsides, depsidones, dibenzofurans, and anthraquinones are unique to lichens and contribute significantly to their ecological and pharmacological properties [13,14]. These compounds are mainly produced by the fungal component and are often accumulated extracellularly as crystalline deposits [17].

The biological importance of lichens has been recognized since ancient times, where they were used in traditional medicine for the treatment of various diseases including respiratory disorders, infections, diabetes, and skin ailments [29]. Modern scientific investigations have confirmed that lichen-derived compounds exhibit a wide range of pharmacological activities such as antimicrobial, antioxidant, anti-inflammatory, antidiabetic, immunomodulatory, and anticancer effects [25,31]. These activities are largely attributed to the presence of phenolic compounds, flavonoids, and other bioactive secondary metabolites. Several studies have reported the antimicrobial potential of lichen species such as Hypogymnia tubulosa, Cetraria aculeata, and Usnea hirta, which produce compounds with strong antibacterial and antifungal activities [225–233] . Similarly, antioxidant and radical scavenging activities have been observed in species containing compounds like usnic acid, gyrophoric acid, and salazinic acid. In addition, lichens have

demonstrated significant enzyme inhibitory activities relevant to diabetes management, including α-glucosidase inhibition. Despite these promising biological properties, lichens remain an underexplored source of natural bioactive compounds due to challenges such as slow growth, difficulty in identification, and limited availability of biomass for large-scale studies [31]. However, advancements in analytical techniques, extraction methods, and biotechnological approaches have opened new avenues for the exploration and utilization of lichen metabolites in drug discovery. Therefore, the present study aims to investigate the phytochemical composition and evaluate the biological activities of selected lichen species. The study focuses on the identification, extraction, and characterization of bioactive metabolites and their potential applications in pharmaceutical research.

Materials and Methods

Collection, identification and extraction of phytochemicals from lichens:

Collection of lichen samples: Lichen species were collected during March 2013 from Ooty, Tamil Nadu mainly at coordinates of 11.4064°N to 11.436160°N and 76.6032° E to 76.696408° E with average altitude of 2,240 meters. No crustose lichen from bark of tree was collected so most of lichens were collected as they were fallen from the tree since they were of foliose and fruticose types. Collected lichen sample were kept in lichen wrap bags and were transferred to laboratory. They were soaked in water and washed to remove their debris and insects, then were dried and some part of it was measured and powdered by grinder for the extraction process. Remaining parts were preserved in acid-free packets for further use.

Identification:

A) Lichen samples were identified on the base of their morphology and chemical characteristics. Lichens were screened for their morphology based on their growth type, presence or absence of vegetative parts (Rhizines and cilia), and sexual reproductive parts (Their types of Apothecia and Perithecia, if any) and color of thallus [5, 245]. Spot tests, TLC and micro-crystallography were chemical methods applied to lichen fragment and their extract to help the identification of species.

B) Spot test: For Calcium/‗C' test, a drop of Ca(OCl)solution, which was prepared freshly applied on lichen fragment. Aromatic compounds with two free-OH- groups react to this solution and form a red color on thallus of lichen [194]. For potassium or ‗K' test, 10-25% aqueous solution of potassium hydroxide is used as the reagent. Quinonoid lichen compounents react to this solution and form a dark red color [194]. For phenyl-enediamine or ‗Pd' test, 1-5% ethanolic solution of p-phenyl-enediamine is used; it reacts with aromatic aldehydes and gives yellow to red color on tested fragment. For KC test, first K solution applied followed by immediate use of C. Some depsides and depsidpnes give red color after applying this procedure [194]. Iodine or ‗I' test also was used by a 0.5% potassium iodide solution which reacts with certain polysaccharides in lichen.

C)Micro-crystallography:

A small piece of each lichen thallus was placed on a slide and lichen substances extracted by drop wise adding crystallizing solution which is glycerol: ethanol: water (1:1:1) [194]. After light heating of slide, they were observed under microscope and photos were compared with reference photograph available in literature like; Culberson, 1969 [246] and 1970 [247,248], Huneck and Yoshimura (1996) [249] and Hale (1974) [195].

D) Thin Layer Chromatography:

Lichen extracts were spotted on heat-activated silica coated TLC Aluminum sheets (Silica gel 60 F254, Merck, Germany) and were run separately by four standard solvents. Four different organic mobile phase used for TLC were as follows: EA (Diethyl ether: Acetic acid; 200 ml: 2 ml); HEF (Hexane: Diethyl ether: Formic acid; 140 ml: 100 ml: 20 ml); TDA (Toluene: 1-4 Dioxane: Acetic acid; 180 ml: 45 ml: 5 ml); TEF (Toluene: Ethyl acetate: Formic acid; 65.5 ml: 41.5 ml: 4 ml). TEF solvent showed better separation of bands so in further study this mobile phase was used as standard solvent. Plates were dried for evaporation of solvents [164]. Then developed bands were visualized under UV chamber and by spraying 10% Sulphuric acid solution. Lichen materials were also sent for authentication Lichenology Lab at CSIR- National Botanical Research Institute (NBRI), Lucknow. A part of each lichen species is deposited as dried herbarium specimen in National Lichen Herbarium, NBRI, Lucknow, India.

1.1) Extraction:

5 grams of each powdered lichen material was kept in handmade thimble and placed in Soxhlet apparatus (Fig-8). Extraction was carried out in various organic solvents because lichen substances are poorly soluble in water [250]. In order to obtain maximum extraction of lichen substances, solvents were used according to increasing polarity [164] as follows: hexane, ethyl acetate, methanol and ethanol (Fig-7). Extraction time for each solvent was about 8 hrs. Concentration of each lichen crude extract was recorded and percentage of yield was calculated. Final obtained yield is a measure of solvent's efficiency to extract specific components from sample [251]. Percentage of yield was calculated by below formula.

Yield (%) = (dry weight of extract/dry weight of sample) x100

Results and Discussion

Qualitative phytochemical screening of all lichen species:

All extracts were subjected to different tests to determine specific groups of compounds available in each extract. Overall, it was observed that methanol extract contained more phytochemicals present in the lichen species. The order of phytochemical harbuored by each lichen species was as follow: P. tinctorum, P. reticulatum, P. crinitum, H. leucomelos, Herpothallon sp., C. subradiata and Leptogium sp. Saponin and cardiac glycosides were rarely present in lichen's crude extracts while they were rich in phenol and flavonoid compounds. Some phytochemical tests with different color reactions are shown in Fig-15. All lichen extracts showed intense flavonoid compounds as qualitatively measured except few ethanolic extracts. Flavonoids were found to be the most widely distributed group of phytochemicals in all lichen extracts, followed by phenols, carotenoids, steroids, carbohydrates, reducing sugars, xanthoproteins, alkaloids, anthraquinones, trepenoids, and other minor present groups.

Quantification of phytochemicals in lichen extracts

Phenolic, flavonoid and carotenoid components were observed in almost all species; therefore, these groups were subjected to

quantify their content in each extract based on individual standard calibration line. Since tested lichen extracts were high in these classes of phytochemicals, their antioxidant content also was quantified. Calibration line of each standard chemical used in this study was constructed and results are presented in Fig-17. From these calibration lines, Concentration of phenolic, flavonoid, carotenoid and antioxidant contents were calculated based on obtained formula from Trend line, where Y is measured O.D. and X is unknown concentration. Results were expressed as mg of each.

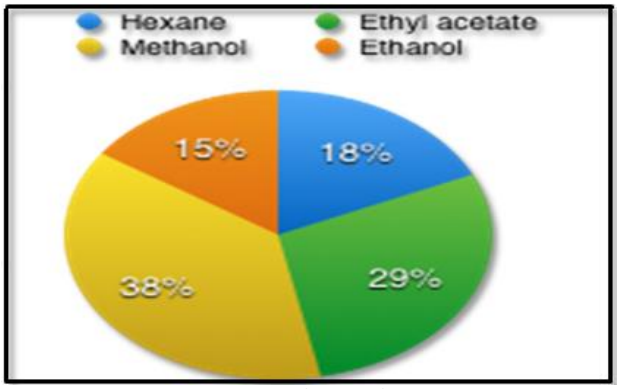

Fig.1: Mass production of extract from each solvent.

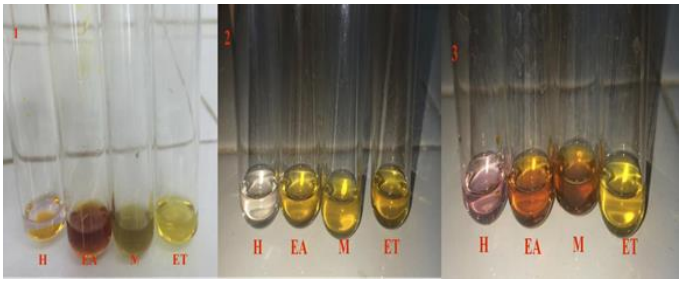

Fig.2: Color changes in some qualitative phytochemical test: 1: Alkaloid (+) in hexane extract of Leptogium sp., 2: Flavonoid (+) in methanol extract of Herpothallon sp., 3: Anthraquinon (+) in methanol extract of H. leucomelos.

Anti-diabetic activity of lichen extracts

In vitro α-amylase inhibitory of lichen extracts

Lichen extracts were subjected to fungal diastase (α-amylase) for screening their anti-amylase activity spectrophotometrically. Investigation carried out on three different concentrations (5, 10 and 15 mg/mL) to find out inhibitory concentration pattern of each species. Results were illustrated in the form of percentage of inhibition and IC50 . In all tested samples, a dose dependent response was observed; higher concentration had more inhibition percentage. Ethyl acetate found to be a good solvent for extraction of amylase inhibitory compound. All lichen extracts in 15 mg/ml indicated moderate to great inhibitory activity ranging from 47.9 % by Leptogium sp. to 99.2 % inhibition in case of *H. leucomelos*. *Lichen H. leucomelos* found to be great amylase inhibitor as only in 5mg/ml, 95.77% inhibition in amylase enzyme was observed Activity order of lichens by enzyme inhibition was as follow: *H. leucomelos, C. subradita, P. reticulatum, P. tinctorum, Leptogium sp., Herpothallon sp., P. crinitum and R. celastri.* This order does not mean that R. celastri as the lowest lichen in this list had low amylase inhibitory action as 15mg/ml of its ethyl acetate extract, had 92.4% inhibition, it's just overall inhibition among all extracts.

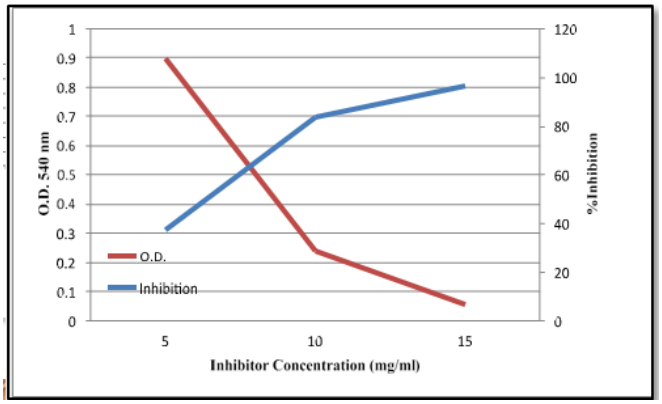

Fig-3: Relationship between turbidity and % inhibition in ethyl acetate extract of P. reticulatum. activity.

All ethanolic lichen extracts showed inhibition from 12% *(P. reticulatum) to 88% (C.subradiata). In ethanol extracts, C.subradiata followed by H. leucomelos and P.crinitum had* shown highest inhibitory activity respectively. Overall highest inhibition was observed in ethyl acetate extract of *R. celastri by 91%. Acarbose (20µg/mL) as positive standard anti- diabetic drug, showed 65%* inhibition, which is much less than *R.celastri*

Discussion

The present study was undertaken to evaluate the phytochemical composition and biological activities of selected lichen species collected from Ooty, Tamil Nadu. The lichens were identified using morphological, chemical (spot tests, TLC, microcrystallization), and chromatographic techniques, ensuring accurate classification. Extraction was performed using solvents of increasing polarity, namely hexane, ethyl acetate, methanol, and ethanol. Among these, methanol yielded the highest extractive value and phytochemical content, indicating its efficiency in extracting bioactive constituents.

Qualitative phytochemical screening revealed the presence of multiple secondary metabolites including alkaloids, flavonoids, phenols, tannins, terpenoids, steroids, carbohydrates, and anthraquinones. Among these, flavonoids and phenolic compounds were the most abundant, while volatile oils were present only in trace amounts. Quantitative analysis further confirmed that methanolic extracts were rich in phenols, flavonoids, and antioxidant content. A strong positive correlation was observed between phenolic and flavonoid content with antioxidant activity, whereas carotenoids showed no significant correlation. The antimicrobial activity of lichen extracts demonstrated broad-spectrum bactericidal effects. Methanolic extracts showed the highest activity, with very low MIC, NIC, and MBC values, indicating strong potency against various pathogens. Growth kinetics studies revealed that lichen extracts reduced microbial growth rate and increased lag phase duration, confirming their inhibitory effect on bacterial proliferation. The antidiabetic potential of lichen extracts was evaluated through α-amylase and β-glucosidase inhibition assays. All extracts exhibited dose-dependent enzyme inhibition. Notably, Heterodermia leucomelos showed the highest α-amylase inhibition (99.2%) with a very low IC_{50} value, while Ramalina celastri exhibited strong β-glucosidase

inhibitory activity. These findings indicate that lichens can serve as potential natural agents for the management of diabetes mellitus. Anticancer activity assessed by MTT assay demonstrated that lichen extracts exhibited selective cytotoxicity against cancer cell lines with minimal effect on normal cells. Among all species, Parmotrema crinitum showed the highest cytotoxicity with a very low IC_{50} value. Further mechanistic studies revealed that the extract induced early apoptosis and complete G1 phase cell cycle arrest, indicating a strong antiproliferative effect. Isolation and characterization of bioactive compounds from P. crinitum led to the identification of stictic acid, constictic acid, and a novel anthraquinone (1-hydroxy-3-methoxy-6-methylanthraquinone). These compounds were found to contribute synergistically to the biological activities of the extract. Overall, the study highlighted that lichens are a rich source of bioactive metabolites with significant pharmacological potential.

Conclusion

The findings of the present study clearly demonstrate that lichen species possess remarkable pharmacological properties due to their rich phytochemical composition. Methanolic extracts were found to be the most effective in extracting bioactive compounds, particularly phenols and flavonoids, which play a crucial role in antioxidant activity. The study confirmed that lichen extracts exhibit strong antimicrobial activity with bactericidal effects, making them promising candidates for the development of natural antibiotics. Their ability to alter microbial growth kinetics further supports their therapeutic potential. Significant antidiabetic activity was observed through effective inhibition of α-amylase and β-glucosidase enzymes, suggesting that lichens can be developed as natural agents for controlling hyperglycemia with potentially fewer side effects. The anticancer potential of lichens, especially Parmotrema crinitum, was one of the most important findings of this study. The induction of early apoptosis and complete G1 phase arrest indicates a targeted mechanism of action against cancer cells while sparing normal cells. The isolation of bioactive compounds, including a novel anthraquinone, further strengthens the importance of lichens as a source of new drug candidates. The observed synergistic activity among isolated compounds highlights their enhanced therapeutic efficacy in natural form. In conclusion, lichens represent a valuable and underexplored source of natural bioactive compounds with diverse therapeutic applications, including antimicrobial, antioxidant, antidiabetic, and anticancer activities. These findings support further in vivo studies, clinical evaluation, and drug development efforts to utilize lichens as potential pharmaceutical agents.

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Conflict of Interests

The authors declare no conflict of interest

Ethics Approval: Not applicable

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AI Tool Declaration

The authors declare that no AI and related tools are used to write the scientific content of this manuscript.

Data Availability

Data will be available on request

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